Filaggrin Mutations in Children with Severe Atopic Dermatitis

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Atopic dermatitis (AD) results from strong genetic and environmental interactions. AD shows genetic linkage to Chromosome 1q21. This region contains the epidermal differentiation complex (EDC), which consists of genes that form essential components of epidermal surfaces. Filaggrin *(FLG)* is one of these. Mutations in *FLG*/(R501X and 2282del4) are reported to be strongly associated with AD and to influence asthma accompanying AD. We investigated these effects in families recruited through a child with severe AD. We genotyped two panels of families, totalling 426, containing 990 affected and unaffected children. We found significant associations with AD ($P=0.0001$), asthma ($P=0.006$), and atopy ($P=0.002$). The *FLG* mutations were present in 26.7% of patients with AD, but were also present in 14.4% of children without AD. They were weakly associated with disease severity. The variants were not independently associated with asthma. The overall LOD score for genetic linkage of markers to the region was 3.57. This fell to 2.03 after accounting for the *FLG* mutations, indicating the presence of other genetic variants influencing AD at this locus. Our results provide further confirmation of the importance of mutations in *FLG* and the skin barrier in AD pathogenesis. The results indicate that investigations of other genes within the EDC should be undertaken.

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INTRODUCTION

Atopic dermatitis (AD) is strongly genetic (Larsen *et al.*, 1986; Schultz Larsen, 1993), and the discovery of genes underlying the disease offers the promise of better understanding of its pathogenesis. Genome screens have identified areas of genetic linkage to AD on Chromosomes 1q21 (Cookson *et al.*, 2001), 3q21 (Lee *et al.*, 2000; Bradley *et al.*, 2002), 3p24–22 (Bradley *et al.*, 2002), and 17q25 (Cookson *et al.*, 2001). The composite phenotype of AD and asthma combined has also shown significant linkage to Chromosome 20p (Cookson *et al.*, 2001). The 1q21, 3q21, 17q25, 20p, and 5q31 loci overlap with psoriasis loci (Cookson *et al.*, 2001; Bowcock and Cookson, 2004).

Chromosome 1q21 is of particular interest as it harbors the epidermal differentiation complex (EDC). This is a group of genes and gene families that are physically contained within 1.8 Mb of DNA and are expressed during terminal differentiation of the human epidermis. Gene families within the EDC include the S100 calcium binding proteins, small proline-rich proteins, and late expressed cornified envelope proteins (Mischke *et al.*, 1996; Marshall *et al.*, 2001). Members of the peptidoglycan recognition protein family (*PGLYRP3* and *PGLYRP4*) are also encoded within the region. Single copy genes in the EDC include trichohyalin, repetin, involucrin, filaggrin (*FLG*), and loricrin.

Monogenetic disorders have successfully been used to identify genes in AD. Mutations in *SPINK5* cause Netherton syndrome in which atopy is a universal accompaniment. Variants in *SPINK5* provide susceptibility to AD (Walley *et al.*, 2001; Kato *et al.*, 2003; Nishio *et al.*, 2003; Kusunoki *et al.*, 2005), though no association was seen in one study (Folster-Holst *et al.*, 2005). These associations with *SPINK5* mutations and variants support the hypothesis that a dysfunctional skin barrier predisposes to AD (Cookson, 2004).

Another recessive cutaneous disorder, ichthyosis vulgaris, has recently been shown to be caused by loss-of-function homozygous or compound heterozygous mutations, R501X and 2282del4, in exon three of the *FLG* gene (Smith *et al.*, 2006). Approximately 8% of patients with AD have clinical features of ichthyosis vulgaris (Tay *et al.*, 1999). Immunohistochemistry, ELISA (Seguchi *et al.*, 1996), and microarray (Sugiura *et al.*, 2005) studies have previously shown decreased protein levels and decreased RNA expression of *FLG* in AD skin.

Profilaggrin is found in the keratohyalin granules in the stratum granulosum. Profilaggrin is dephosphorylated and proteolytically cleaved to *FLG* during terminal differentiation of the granular cells. *FLG* then aggregates the keratin filaments, which collapse the granular cells into anuclear squames. The cytoskeleton is then crosslinked by trans-

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Abbreviations: AD, atopic dermatitis; CI, confidence interval; EDC, epidermal differentiation complex; FLG, filaggrin; OR, odds ratio

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glutaminases to form the cornified cell envelope of the stratum corneum (Markova *et al.*, 1993) and the outermost barrier of the skin. *FLG* is therefore an excellent candidate gene for AD.

The group that identified the two *FLG* mutations underlying ichthyosis vulgaris subsequently found them to be strongly associated with AD and asthma in populations of European origin (Palmer *et al.*, 2006). Their results indicated that the – variants were carried by ~9% of people of European origin (Palmer *et al.*, 2006). In collaboration with this group, investigators also found these loss-of-function variants to be associated with AD and allergic sensitizations in a German cohort and trios (Weidinger *et al.*, 2006) and with adult AD (Barker *et al.*, 2006). In the German samples the *FLG* mutations were associated with extrinsic AD characterized by raised IgE and allergic sensitizations and not the intrinsic form of the disease (Weidinger *et al.*, 2006). Another German group also independently found *FLG* mutations to be strongly associated with AD, although only the R501X mutation was tested in their panel of trios (Ruether *et al.*, 2006). A significant association with *FLG* null mutations with AD has also been shown recently by a German group that investigated families and population-based cases and controls (Marenholz *et al.*, 2006).

These results all indicate that mutations in *FLG* are a significant risk factor for AD. It claims, however that they are also an independent risk factor for AD-associated asthma (Palmer *et al.*, 2006; Weidinger *et al.*, 2006) and that 50% or more of children with moderate to severe AD and 20% of all cases of asthma associated with AD carry *FLG* mutations (Irvine and McLean, 2006) suggest additional hypotheses that do require investigation.

We have therefore investigated the two *FLG* variants in panels of families recruited through children with AD from a tertiary referral center. These children represent the most severe end of the disease spectrum and form an important clinical group who are often resistant to maximal therapy. Approximately 60% of these children also suffer from asthma. These families contained affected as well as unaffected children, allowing us to examine the effect of *FLG* mutations in normal and diseased subjects sharing a common genetic and environmental background.

RESULTS

In the ECZ1 panel, 254 children had AD, with a mean age of 6.89 years (±SEM 0.270), 124 (48.8%) were male subjects, 139 (55.6%) had asthma, and the mean total serum IgE in children with AD was 438.6 IU/L (95% confidence interval (CI) = 328.5–585.5). In the replication panel MRCE, 403 children had AD. The children's mean age was 6.7 years (±SEM 0.21). 223 (55.3%) were male subjects and 233 (57.8%) had asthma. Their mean total IgE was 521.5 IU/L (95% CI = 328.5–585.5). In all 18.9% had mild disease, 52.1% had moderate disease, and 29% had severe disease in the ECZ1 panel. In the MRCE panel, 15% were mild, 46.2% were moderate and 38.8% were severe. All children however were on maximal therapy when recruited. With regard to ethnicity in the ECZ1 panel, the three major groups were Caucasian (90%), Asian (1%), and mixed race (4.3%). In the MRCE panel, 69% were Caucasian, 14% South Asian, and 8% were of mixed race.

Hardy–Weinberg equilibrium was observed for both markers. We initially tested the two family panels independently for association with AD, before combining the panels in subsequent tests. We similarly investigated the two mutations separately (to determine if they had differing effects on disease susceptibility), as well as testing for association with a combined genotype for both mutations. As association between AD and *FLG* mutations is not in doubt, uncorrected *P*-values for association testing are given throughout.

Significant associations were seen in affected children in the ECZ1 panel for the single-nucleotide polymorphism R501X with AD (T 24, NT 10, and $P = 0.02$) and for 2282del4 for AD (T 39, NT 13, and $P = 0.0003$), asthma (T29, NT10, and $P = 0.002$), and atopy (T 37, NT 13, and $P = 0.0007$). For the combined genotype associations were seen for AD (T 64, NT 33, and $P = 0.002$), asthma (T 41, NT 24, and $P = 0.04$), and atopy (T 58, NT 33, and $P = 0.009$) using the transmission disequilibrium test.

In the MRCE panel significant associations were seen with R501X and AD (T 55, NT 21, and $P = 0.0001$), asthma (T 43, NT19, and $P = 0.002$), and atopy (T 57, NT 27, and $P = 0.001$) and for 2282del4 for AD (T 30, NT 16, and $P = 0.04$), asthma (T 26, NT 12, and $P = 0.02$), and atopy (T 35, NT20, and $P = 0.04$). For the combined genotype associations were seen for AD (T 84, NT 53, and $P = 0.008$) and atopy (T88, NT 64, and $P = 0.052$).

Associations were also seen in the combined family panels for R501X with AD ($P = 0.0001$), asthma ($P = 0.0008$), and atopy ($P = 0.0002$). For the 2282del4 marker associations were seen with AD ($P = 0.0001$), asthma ($P = 0.0002$), and atopy ($P = 0.0001$) and for the combined genotype for AD ($P = 0.0001$), asthma ($P = 0.006$), and atopy ($P = 0.002$), (Table 1). The results were not different in terms of transmission frequencies when only Caucasians were examined.

In order to investigate the penetrance of the mutations, transmission ratios were also examined in unaffected children (Table 1). Both variants were common in the unaffected children, confirming that the mutations are not exclusively associated with AD.

The distribution of *FLG* variants was then examined in children with and without AD. The *FLG* null variants were present in 26.7% of children with AD and in 14.1 % of unaffected children. We therefore calculated the penetrance of AD in *FLG* null carriers to be 78.7% in these subjects.

We investigated the risk of disease in affected and unaffected children using logistic regression analysis. Age and sex did not significantly predict the presence of AD in the logistic model, perhaps reflecting the narrow age range and sex ratios of children in our study. The exclusion of non-Caucasians did not affect the estimates of risk of disease either. Dominant or recessive models did not fit the data better than an additive model.

The results indicated that the combined genotype was associated with an increased risk (odds ratio (OR) 2.03, 95%

Table 1. Results for affected and unaffected children in the combined family panel for *FLG* mutations R501X, 2282del4, and the combined genotype

SNP	AD affected T:NT[1] (*P*)	Asthma affected T:NT (*P*)	Atopy affected T:NT (*P*)	AD unaffected T:NT (*P*)	Asthma unaffected T:NT (*P*)	Atopy unaffected T:NT (*P*)
FLG501X	79:31 (0.0001)	55:25 (0.0008)	76:36 (0.0002)	21:30 (NS)	44:34 (NS)	23:22 (NS)
FLG2282del4	69:29 (0.0001)	55:22 (0.0002)	72:33 (0.0001)	18:21 (NS)	30:30 (NS)	19:12 (NS)
Combined	148:86 (0.0001)	108:71 (0.006)	146:97 (0.002)	59:36 (NS)	75:73 (NS)	48:35 (NS)

[1]FLG, filaggrin; T, number of transmitted alleles; NS, not significant; NT, number of untransmitted alleles.

Table 2. Logistic regression analysis of association between *FLG* mutations and AD in all children

	P-value	OR	95% CI
FLG501X	0.001	2.55	(1.50–4.34)
FLG2282del4	0.004	1.93	(1.23–3.03)
Compound genotype	<0.001	2.03	(1.46–2.81)

AD, atopic dermatitis; CI, confidence interval; FLG, filaggrin; OR, odds ratio.

Table 3. Logistic regression analysis of association between *FLG* mutations (compound genotype) with AD in atopic and non-atopic children

	P-value	OR	95% CI
Atopic children	<0.001	2.28	(1.46–3.56)
Non-atopic children	0.45	1.29	(0.67–2.49)

AD, atopic dermatitis; CI, confidence interval; FLG, filaggrin; OR, odds ratio.

Table 4. Contingency table for logistic regression analysis of genetic predictors of association between *FLG* mutations (compound genotype) and asthma in individuals without AD

	11	12	22	*P*-value	OR	95% CI
Full sample, no asthma	589	111	6			
Full sample, asthma	122	24	0	0.78	0.94	(059–1.48)
Children only, no asthma	202	29	3			
Children only, asthma	47	11	0	0.49	1.28	(0.64–2.55)

AD, atopic dermatitis; CI, confidence interval; FLG, filaggrin; OR, odds ratio; 11, wild-type allele; 12, heterozygous for either mutation; 22, homozygous for either mutation or heterozygous for both mutations.

CI = 1.5–2.8) for developing disease (Table 2). Calculation of the Pseudo-R^2 for the regression model (Nagelkerke 1991), indicated that the *FLG* mutations contributed 3.2% to the variation of the presence of AD in these children. This suggested that other genetic and environmental factors, in addition to the *FLG* mutations, were necessary for the development of disease in these subjects.

We then examined the relationship of the *FLG* mutations to AD separately in atopic and non-atopic children. Logistic regression analysis showed the *FLG* variants to be associated with extrinsic AD characterized by atopy (OR 2.28, 95% CI = 1.46–3.56), (Table 3). When non-atopic children were examined, there were no significant associations with AD.

We next performed a logistic regression to determine the relationship of the compound mutation genotype to asthma in the absence of AD (Table 4). There were 58 asthmatics and 234 non-asthmatics amongst the children without AD. This gave 96% power to detect a gene with an OR of 2 for disease for each risk allele with $\alpha = 0.1$ and 93% power with $\alpha = 0.05$ (Purcell *et al.*, 2003). The mutations were not significantly associated with asthma in these children (OR 1.28, 95% CI = 0.64–2.55, and $P = 0.49$).

Including parents in the analyses, there were 146 asthmatics and 706 non-asthmatics amongst individuals without AD. This gave 99.98% power to detect a gene with an OR of 2 for each disease allele with $\alpha = 0.05$, and 98% power to detect a gene with an OR of 1.5 at $\alpha = 0.05$. The mutations also did not show evidence for association to asthma in this full sample (OR 0.94, 95% CI = 0.59–1.48, and $P = 0.78$).

The *FLG* mutations were significantly associated with the severity of AD (measured by the criteria of Rajka and Langeland (1989)), with $P = 0.007$ in a linear regression model. The adjusted R^2 was however 0.8%, indicating a minor effect on the severity of the illness in these subjects. Calculation of variance in severity owing to *FLG* mutations within the quantitative transmission disequilibrium test program gave a similar result of 0.9%.

We next examined if the *FLG* mutations accounted for the previously reported linkage signal between the EDC microsatellite marker *D1S498* and AD (Cookson *et al.*, 2001). We reanalysed the data for the previously reported highly linked marker *D1S498* (Cookson *et al.*, 2001) with the compound *FLG* genotype in the ECZ1 panel of families. We used the LAMP program (Li *et al.*, 2005) which maximizes the evidence for association across all genetic models, and provides tests for the presence of additional linked variants in addition to the mutation under study. For AD, the evidence for linkage to the markers was a total LOD score of 3.57 (3 degrees of freedom (df)), $P = 0.0009$. The *FLG* only LOD

score was 1.54 and the residual evidence for linkage after the *FLG* mutations were taken into account was a LOD score of 2.03 (2 df), $P=0.009$, indicating the presence of other genetic variants influencing AD in proximity to *D1S498* and the EDC. These variants may include as yet undiscovered mutations in *FLG* itself, or polymorphisms in other epidermal genes from the locus.

DISCUSSION

The results support prior studies in indicating that the *FLG* mutations have an important influence on susceptibility to AD (Barker *et al.*, 2006; Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Ruether *et al.*, 2006; Weidinger *et al.*, 2006). Our multivariate analyses confirm that the variants are strongly but incompletely penetrant (Palmer *et al.*, 2006).

Our results indicate that the *FLG* mutations did not have an independent effect on asthma, at least in our children with severe AD and their families. *FLG* is not expressed in the lung mucosa, and the suggestion that a predisposition to asthma may result from bystander allergies secondary to a permeable skin (Hudson, 2006) represents a departure from current understanding of asthma pathogenesis that requires further investigation.

We found a higher frequency of *FLG* variants in unaffected siblings from our family panels than previously reported population estimates (Palmer *et al.*, 2006). This is attributable to the relatedness of these individuals to their siblings with AD. Although ORs calculated in families are not directly comparable to case–control studies, the risk of AD conferred by *FLG* mutations in children with AD and their unselected siblings appeared lower than might be expected from previous reports of ORs ranging from 2.73 to 13.4 (Barker *et al.*, 2006; Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Ruether *et al.*, 2006; Weidinger *et al.*, 2006). In addition, our allele transmission frequency of 63% for AD for the combined genotype, was lower than previous family studies (Marenholz *et al.*, 2006; 68%; Weidinger *et al.*, 2006; 73%)).

The reason for the apparently reduced risk in our subjects is not clear. Some random differences between population samples are to be expected in the study of any complex genetic disease. The use of unselected siblings as controls implies that we have studied the effect of *FLG* mutations in individuals that share a common environment and genetic background to affected children, providing a level of control for these factors. Occult population admixture may also confound estimations of risk, and a proportion of families in our study were of South Asian origin. We found that exclusion of these subjects did not alter our estimates of risk or our observed transmission ratios to affected individuals. The use of family-based association testing has provided an additional level of control for the effects of admixture.

Differences in disease risk between studies could also be accounted for by variation in disease spectrum. It may be relevant that we have studied young children with severe AD presenting to a tertiary referral center. In the context of health care in the UK, tertiary referral is usually an indicator of previous therapeutic failure. The therapy-resistant children in our study may therefore have had AD that was driven by a different balance of risk factors to population-based cases. The observation that *FLG* mutations are extremely high-risk factors for patients with persistent adult disease (Barker *et al.*, 2006) is also consistent with an underlying heterogeneity of the disorder.

Both *FLG* variants that we tested are largely confined to Europeans, and they have been found to be absent in non-European populations of African and Asian origin (Palmer *et al.*, 2006). A third loss-of-function variant in *FLG* has recently been identified, which highlights the variability of this gene (Sandilands *et al.*, 2006), but no common mutations have yet been identified in non-Caucasian populations. Population based studies have shown a 2-fold higher prevalence of AD in black Caribbean children compared to Caucasians children born in London (Williams *et al.*, 1995). A similar AD prevalence has been reported when black infants of West Indian origin were compared to Caucasians living in London (Davis *et al.*, 1961). Further studies are required to identify the role of this gene in other ethnicities and the extent to which AD may be genetically heterogeneous in such populations.

Our results suggest that the *FLG* mutations are not solely responsible for the significant genetic linkage of AD to the EDC region on Chromosome 1q21 reported previously in the ECZ1 panel (Cookson *et al.*, 2001). The genes of the EDC are expressed late during terminal differentiation and maturation of keratinocytes. Genes from the EDC have been associated with other cutaneous Mendelian disorders, including Vohwinkel's syndrome (Maestrini *et al.*, 1996), and other genes in the epidermal barrier have been shown to contribute to atopic disease (Walley *et al.*, 2001).

Many of the EDC genes may be candidates for AD by virtue of their function and location. For instance, involucrin, loricrin, and small proline-rich proteins encode structural proteins that with trans-glutaminase mediate cross linking resulting in the formation of the cornified cell envelope (Roop, 1995). The S100 proteins are a multigene family of low molecular weight calcium-binding proteins that have a wide variety of functions that regulate the epidermal response to tissue injury, inflammation, and disease (Kerkhoff *et al.*, 1999). They are increased in response to a variety of skin stresses such as tape stripping and UV exposure (Gebhardt *et al.*, 2002). Interestingly in a microarray study of adult AD patients versus controls *S100A2*, *S100A7*, *S100A8*, and *S100A9* levels were all found to be upregulated whereas the cornified envelope genes loricrin and *FLG* were downregulated (Sugiura *et al.*, 2005).

Additionally, peptidoglycan recognition proteins 3 and 4 (*PGLYRP3* and *PGLYRP4*) are members of a family of innate immunity pattern recognition proteins and are bactericidal against Gram-positive bacteria. Several single-nucleotide polymorphisms in the *PGLYRP3-PGLYRP4* locus have recently been reported to show association with psoriasis in a family based analysis using the transmission disequilibrium test (Sun *et al.*, 2006). More than one-third of AD susceptibility loci map to psoriasis linkage peaks (Cookson *et al.*, 2001; Bowcock and Cookson, 2004) suggesting that these

two cutaneous conditions share genes or gene complexes that are specific to cutaneous barrier defences and immunity.

The presence of numerous candidate genes in addition to *FLG* within the EDC suggests that further investigation of this complex locus may identify other genetic variants with effects on a spectrum of dermatological disorders.

MATERIALS AND METHODS

Families were recruited from the Dermatology clinics at the Great Ormond Street Hospital for Children, through a child or children with active AD. A physician-administered questionnaire was completed for each individual. The questionnaire included the modified Hanifin and Rajka diagnostic criteria for AD defined by the UK Working party (Williams *et al.*, 1994a, b) and questions based on the American Thoracic Society's questionnaire for asthma and allergic rhinitis. Each family was examined for evidence of AD by a physician. Children lacking signs of disease were classified as unaffected. The severity of eczema was assessed using the scoring system of Rajka and Langeland (1989), which categorizes patients into mild (3.0–4.0), moderate (4.5–7.5), or severe (8.0–9.0) disease on the basis of surface area involvement, continuity of disease, and nocturnal pruritus (Rajka and Langeland, 1989). Asthma was defined on the basis of the questionnaire answers and a previous physician diagnosis as described previously (Cox *et al.*, 1998). Skin prick tests (Dome-Hollister-Stier, Spokane, WA) were carried out on all individuals for a range of allergens including: house dust mite (*Dermatophagoides pteronyssinus*), Timothy Grass (*Phleum pratense*), mould (*Alternaria alternata*), cat dander (*Felis domesticus*), egg white and cow's milk.

Total IgE and specific IgE (to the same panel of allergens) was measured by a fluorescent enzyme immunoassay (Pharmacia CAP system, Pharmacia, Uppsala, Sweden). A raised total serum IgE was taken to be greater than 100 kU/L. Atopy was defined as: (i) the presence of a positive skin prick test response 3 mm or greater than the negative control, (ii) a positive specific IgE, (iii) raised total serum IgE, or (iv) any combination of these features.

Initially DNA samples from a panel of 148 nuclear families of northern European origin were examined, the ECZ1 panel. ECZ1 has 350 siblings and 245 sibpairs. The same families had been used previously in an AD genome screen (Cookson *et al.*, 2001). For the purposes of replication, a second eczema panel, the MRC-E eczema panel comprising of 278 families, 634 siblings, and 470 sibpairs was used. Approval for the studies was obtained through the University of Oxford and Great Ormond Street Hospital Ethics Committees. Participants gave their full written informed consent. The investigations were conducted according to the Declaration of Helsinki Principles.

Genotyping for R501X was performed using a Taqman-based allelic discrimination assay (Applied Biosystems, CA). Primers and 6-6-carboxy-fluorescein and VIC-labelled probes were genotyped following standard protocols for the Applied Biosystems 7,300 sequence detection system. Primers and probes used were identical to those previously published (Palmer *et al.*, 2006).

The 2282del4 mutation was genotyped by sizing a 6-carboxy-fluorescein-labelled PCR product on an Applied Biosystems 3,730 DNA sequencer. The wild-type allele was 199 bp and the 2282del4 allele was 195 bp. Primers DEL4F2 and DEL4R1 (Palmer *et al.*, 2006) with 0.05 U/μl AmpliTaq Gold DNA polymerase, 1X buffer, 200 μM dNTP, and 1.5 mM $MgCl_2$ were used in 10 μl PCR reactions. The cycling conditions were: 94°C (12 minutes), one cycle; 94°C (15 seconds), 58°C (30 seconds), and 72°C (45 seconds), 30 cycles; and a final 72°C (5 minutes). PCR fragments were sized against a ROX-500 size marker using standard protocols from Applied Biosystems.

Genotyping was performed blind to the phenotype and genotypes were double checked by two independent investigators (NM and MFM). Hardy–Weinberg equilibrium was tested by using the χ^2-goodness-of-fit test. Family-based association analysis was carried out using the transmission disequilibrium test and quantitative transmission disequilibrium test (Spielman *et al.*, 1993; Abecasis *et al.*, 2000) to examine the transmission rates of marker alleles from parents to offspring for the phenotypes AD, asthma, and total log IgE. Logistic regression analysis of predictors of AD and asthma was performed with SPSS version 14.0. Genotypes were coded as 0 (1 1 homozygous for wild-type allele), 1 (1 2 heterozygous for mutation), and 2 (2 2 homozygous for mutant allele). Dominant models were encoded as 0, 1, and 1 and recessive models were encoded as 0, 0, and 1. Age and sex were also tested for effects in the models.

The status of a residual linkage signal after the *FLG* mutations were taken into account was investigated using the LAMP program (Li *et al.*, 2005). Association and linkage was tested to the compound *FLG* mutation genotype in the ECZ1 panel of families, with *D1S498* used as a framework marker as instructed (http://www.sph.umich.edu/csg/abecasis/LAMP/).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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